

Osmoregulated periplasmic glucans synthesis gene family of *Shigella flexneri*

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Abstract Osmoregulated periplasmic glucans (OPGs) of food- and water-borne enteropathogen *Shigella flexneri* were characterized. OPGs were composed of 100% glucose with 2-linked glucose as the most abundant residue with terminal glucose, 2-linked and 2,6-linked glucose also present in high quantities. Most dominant backbone

polymer chain length was seven glucose residues. Individual genes from the *opg* gene family comprising of a bicistronic operon *opgGH*, *opgB*, *opgC* and *opgD* were mutagenized to study their effect on OPGs synthesis, growth in hypo-osmotic media and ability to invade HeLa cells. Mutation in *opgG* and *opgH* abolished OPGs biosynthesis, and mutants experienced longer lag time to initiate growth in hypo-osmotic media. Longer lag times to initiate growth in hypo-osmotic media were also observed for *opgC* and *opgD* mutants but not for *opgB* mutant. All *opg* mutants were able to infect HeLa cells, and abolition of OPGs synthesis did not affect actin polymerization or plaque formation. Ability to synthesize OPGs was beneficial to bacteria in order to initiate growth under low osmolarity conditions, in vitro mammalian cell invasion assays, however, could not discriminate whether OPGs were required for basic aspect of *Shigella* virulence.

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Introduction

Under low nutrients and low osmolarity growth conditions, bacteria from *Enterobacteriaceae* synthesize a short-chain glucose polymer called glucans that is localized in the periplasmic space (Kennedy 1982; Miller et al. 1986). Synthesis of glucans is osmotically regulated and hence the name osmoregulated periplasmic glucans or OPGs (Hanouille et al. 2004). When cells are subjected to low osmotic stress, the osmolarity of the periplasm that is estimated around 350 mosmol^{-1} prevents the swelling and rupturing of cytoplasmic membrane (Stock et al. 1977; Kennedy 1982). This function is mainly attributed to

anionic short glucose chains, referred in the literature as membrane-derived oligosaccharides (MDOs) or OPGs (Kennedy 1996; Bohin and Lacroix 2007).

We recently demonstrated that OPGs of *Salmonella* are necessary for growth under hypo-osmotic conditions such as vegetable wash waters and are also needed for optimal mice virulence (Bhagwat et al. 2009; Liu et al. 2009). However, *opg* defective mutants of *Salmonella* also showed pleiotropic phenotypes such as reduced motility under low osmotic growth conditions. But lack of motility per se may not be responsible for the compromised virulence since motility revertants of *Salmonella opgGH* remained defective in mice virulence (Kannan et al. 2009). *Shigella* being a nonmotile enteropathogen closely related to *Salmonella* and *E. coli* offers an excellent model system to examine the role of OPGs. In this study, we characterized OPGs of *S. flexneri* and determined the role of individual genes in the OPG gene family by examining the effect of individual mutations on growth in hypo-osmotic media and HeLa cell invasion assays.

Materials and methods

Bacterial strains, growth conditions and media

Shigella flexneri 2a wild-type strain 2457T (Kotloff et al. 1995) was routinely grown on LB media and Tryptic Soy Agar plates containing 0.05% Congo Red (w/v). *Salmonella enterica* serovar Typhimurium SL1344 and its *opg* mutant strains, SG111 and SL_GH3, have been described before (Bhagwat et al. 2009; Kannan et al. 2009). Where appropriate, ampicillin ($100 \mu\text{g ml}^{-1}$), chloramphenicol ($25 \mu\text{g ml}^{-1}$) were added to growth media for selection. *Escherichia coli* strains carrying plasmids pKD3 and pCP20 (Datsenko and Wanner 2000) were obtained from *E. coli* Genetic Stock Center, Yale University. The plasmid pKM208 was obtained from Kenan Murphy (Murphy and Campellone 2003). *S. flexneri* containing plasmids pCP20 or pKM208 were incubated at 30°C as they carry temperature sensitive replicons.

The growth rates of wild-type and *opgGH* mutants were determined in different growth media such as LB and LB with no salts (LBNS) (osmolarity of 407 ± 4 and $85 \pm 4 \text{ mosmol l}^{-1}$, respectively). Osmolarity of growth media was measured with Wescor vapor pressure osmometer (model 5500, Wescor, Inc., Logan UT). LBNS broth diluted 1:8 in distilled water was used as low-nutrient no-salt (LNNS) medium ($36 \pm 3 \text{ mosmol l}^{-1}$). Growth was measured using a Bioscreen C automatic turbidometric analyzer (GrowthCurves USA, NJ) as described earlier (Bhagwat et al. 2009; Liu et al. 2009). Growth was analyzed at 37°C with continuous shaking. To assess the effect

of osmotic stress on growth, media were supplemented with varying amounts of salt (NaCl or KCl) or buffered with HEPES (50 mM, pH 7.1).

Mutagenesis protocol

Mutagenesis was performed using lambda-red protocol as described earlier (Ranallo et al. 2006). Primers were designed based on reference genome sequence of *S. flexneri* 2a strain 2457T (GenBank accession number AE014073). The primers used for mutagenesis of *opgGH*, *opgB*, *opgC* and *opgD* are shown in the supplemental information (Supplemental Table 1). After initial PCR, screen mutations were confirmed by determining DNA sequence flanking individual genes (Supplemental Table 2).

To clone the *opgGH* genes, 4.51-kbp region flanking 426-bp upstream and 22-bp downstream region at the *opgGH* locus were amplified using primers G10 and C12 using Phusion Fidelity PCR kit (New England Biolabs, Ipswich, MA) following manufacturers' instructions. The 4.51-kbp fragment was cloned in pGEM-T vector to get pBP16. The *opgGH* genes were cloned in a low-copy pACYC177-based vector (pPQSL_2.0) to get pLL3. pLL3 was mobilized to *opgGH* mutants by electroporation for complementation studies.

Isolation, purification and characterization of OPGs

To maximize the yield of OPGs, cells were grown in LBNS medium ($85 \pm 4 \text{ mosmol l}^{-1}$) and OPGs were extracted by the charcoal adsorption procedure and were eluted with aqueous pyridine as described (Lequette et al. 2004; Bhagwat et al. 2009). After lyophilization, crude OPGs were subjected to gel filtration using a BioGel P-4 column ($1.5 \text{ cm} \times 100 \text{ cm}$). The column was eluted at room temperature with 7% 1-propanol, and OPGs-containing fractions were monitored by determining the presence of hexose using the phenol–sulfuric acid assay (Dubois et al. 1956). The peak fractions containing the highest amounts of hexose were pooled and lyophilized to isolate the total OPGs. The charged OPGs were further fractionated from total OPGs by ion exchange chromatography using a DEAE-cellulose (Whatman DE53, Sigma Chemicals, St. Louise, MO) column and eluted in a step-wise gradient of 0.05, 0.1, 0.15, 0.2 and 0.5 M NaCl. The unbound fraction was referred to as neutral OPGs.

Glycosyl composition, glycosyl linkage methylation analysis and succinate contents of OPGs

Glycosyl composition analysis was performed at the Complex Carbohydrate Research Center, Athens, GA.

Composition was determined by combined gas chromatography/mass spectrometry (GC/MS) of the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from the samples by acidic methanolysis (York et al. 1985; Merkle and Poppe 1994). For glycosyl linkage analysis, the sample was permethylated, depolymerized, reduced and acetylated; and the resultant partially methylated alditol acetate samples were analyzed by GC/MS (York et al. 1985).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI/TOF-MS) was performed in the reflector positive ion mode using α -dihydroxybenzoic acid (DHBA, 20 mg/ml solution in 50% methanol/water) as a matrix. All spectra were obtained by using a 4700 Proteomics analyzer (Applied Biosystems, USA). Succinate content from OPGs was estimated as described earlier (Lacorix et al. 1999; Bhagwat et al. 2009).

Gentamicin protection assays in HeLa and CaCo-2 cells

HeLa (ATCC CCL-2) infections using *S. flexneri* 2a strains 2457T, 2457T^{CR}, *opgB*, *opgC*, *opgD*, *opgG*, *opgH*, *opgBC* and *opgGH* were performed in accordance with previous literature (Elsinghorst 1994), with the modifications as described earlier (Ranallo et al. 2005). The human CaCo-2 intestinal cell line was obtained from the ATCC (Manassas, VA), and invasion assays using *Salmonella* strains were performed as described earlier (Xia et al. 2009). Intracellular bacteria were plated to LB agar and quantified by the number of colony-forming units (CFU) that appeared after 16-h incubation at 37°C. Rates of invasion were calculated as percentage of wild type, which was set at 100%. Data presented represent the average of three independent experiments. A one-way ANOVA (Prism) was used to test for significant ($P < 0.05$) differences between groups.

Indirect immunofluorescent microscopy of infected HeLa cells

HeLa cells were seeded onto acid-washed glass slides, infected as described above and fixed using 3% formaldehyde for 10 min. Cells were washed in phosphate buffer saline (PBS, pH 7.2) and permeabilized using a solution of 0.2% Triton X-100 for 10 min. Bacteria were stained with guinea pig anti-*S. flexneri* 2a serum and secondary rabbit anti-guinea pig antibodies conjugated with Alexa 488 (Molecular Probes). Filamentous actin was visualized with Texas Red phalloidin (Molecular Probes) and nuclei with 4', 6-diamidino-2-phenylindole (DAPI; Molecular Probes). Confocal microscopy of infected cells was conducted on a Nikon Eclipse E800 upright microscope using a BioRad Radiance 2100 laser module.

Plaque formation in BHK cells

The plaque assay was performed as previously described (Oaks et al. 1985) with modifications as described earlier (Ranallo et al. 2005, 2006). Average plaque sizes for each *Shigella* strain were calculated from at least 10 plaques per 60 mm plate using a Finescale Comparator (Finescale).

LDH determination in infected macrophages

J774A.1 (ATCC TIB-67) macrophages were grown in RPMI 1640 supplemented 10% FBS and 2 mM L-glutamine (cRPMI) and used to seed a 96-well plate. After overnight growth, cells were washed using fresh cRPMI and infected (in triplicate) with log phase cultures of each strain using MOIs of 2.0 and 0.2 as determined by plating and OD₆₀₀ measurements. After the addition of bacteria, the 96-well plate was centrifuged (1,500×g), placed at 37°C and culture supernatants were removed at different times (0, 120, 180 and 240 min) after infection. Wells that did not receive bacteria served as a negative control for spontaneous lysis. LDH activity was measured from freshly harvested supernatants and used to estimate the percent cellular cytotoxicity according to the manufacturer's instructions (Promega, Madison, WI). The assay was performed three times, and the data (% cytotoxicity) were averaged and plotted as a function of time.

Results and discussion

Isolation and characterization of OPGs

Osmoregulated periplasmic glucans from wild-type *Shigella flexneri* 2a strain 2457T were obtained as a single major peak of apparent molecular weight ~1,200 Da from a BioGel P4 gel filtration column (Fig. 1a). No hexose-containing polymers eluted in the void volume. The BioGel P4 elution pattern of OPGs preparations from *opgGH* mutants was devoid of the corresponding peak (Fig. 1a). Synthesis of total OPGs (measured as μ g glucose equivalents per mg cellular protein) was influenced by osmolarity of external growth medium (Fig. 1b). Increasing growth medium osmolarity beyond 300 mosmol l⁻¹ adversely affected OPGs synthesis. Practically, no OPGs were synthesized at medium osmolarity level greater than 440 mosmol l⁻¹. In *E. coli*, *opgGH* encodes a bicistronic operon where OpgH protein exhibits glucosyl transferase activity, catalyzing in vitro elongation of the linear β -1,2 glucose backbone with UDP-glucose as substrate (Kennedy 1996; Debarbieux et al. 1997). OpgG protein is postulated to be involved in the formation of the β 1,6 glucose linkages (Bohin 2000). Other *opg* genes are responsible for

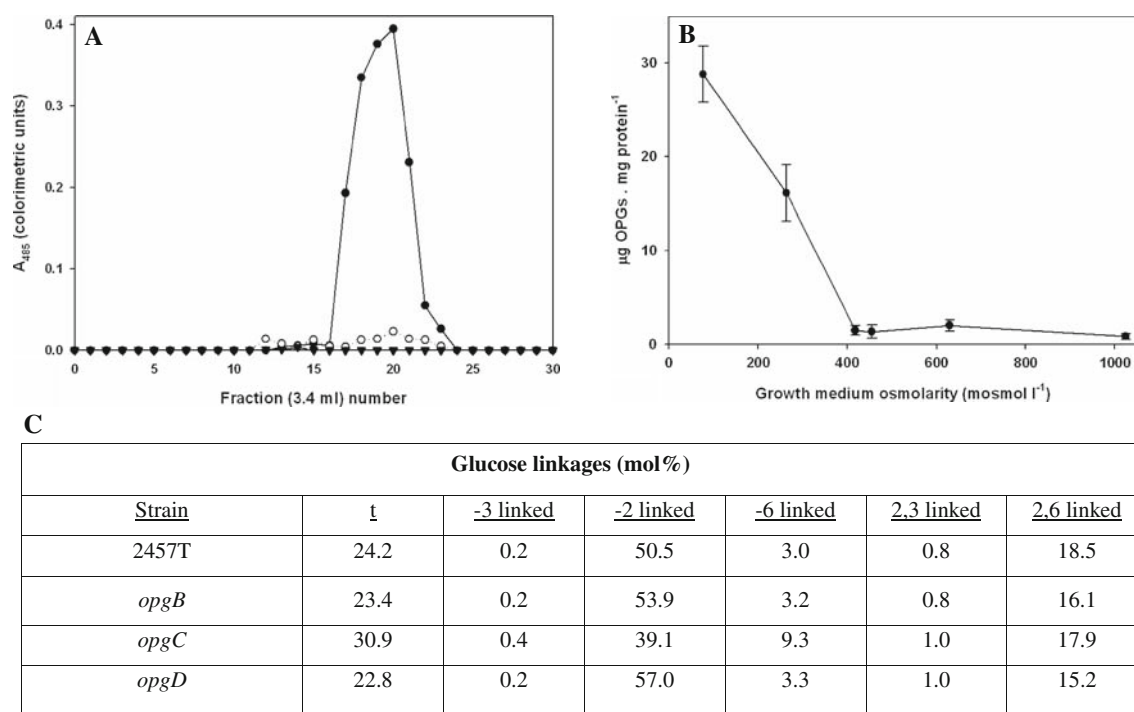


Fig. 1 Characterization of OPGs from *S. flexneri* 2457T. **a** Biogel P4 gel filtration chromatography of OPGs from 2457T (filled circle, filled down pointing triangle) and *opgGH* mutant (open circle). Crude OPGs obtained after pyridine extraction were lyophilized and fractionated using 7% 1-propanol on BioGel P4 column (1.5 by 100 cm). Fractions (3.4 ml) were collected and were analyzed for total hexose by the phenol-sulfuric acid method. Cells were grown in LBNS

(85 mosmol l⁻¹; 2457T, filled circle, and *opgGH*, open circle) or in LBNS ± 0.5 M NaCl (1,038 mosmol l⁻¹; 2457T, filled down pointing triangle). **b** Osmoregulation of OPGs. 2457T cells were grown in LBNS with varying concentration of NaCl (85–1,370 mosmol l⁻¹). OPGs obtained after BioGel P4 chromatography were estimated and plotted as µg OPGs mg⁻¹ cellular protein (filled circle). **c** Linkage analysis of OPGs obtained after BioGel P4 chromatography

succinate (*opgC*) and phosphoglycerol (*opgB*) substitutions of OPGs while *opgD* is predicted to be a paralog of *opgG* and may regulate OPG backbone structure (Kennedy 1996; Lacorix et al. 1999; Lequette et al. 2004; Bohin and Lacroix 2007).

Total OPGs were further analyzed for their hexosyl composition, and glucose was found to be the only component (Fig. 1c). Glycosyl linkage methylation analysis of OPGs indicated that 2-linked glucose was the most abundant residue with terminal glucose, 2-linked and 2,6-linked glucose also present in high quantities (Fig. 1c). Among the *opg* gene family members, only *opgC* mutation influenced overall glycosyl linkage pattern where 2-linked glucose were in reduced proportion with concomitant increase in terminal and 6-linked glucose residues.

A significant portion of the OPGs synthesized was anionic with only ~1% OPGs being neutral and did not bind to DEAE-cellulose (Fig. 2a). Succinic acid contents of the total OPGs from 2457T, *opgB* and *opgD* strains were estimated to be 0.69 ± 0.14 , 0.61 ± 0.21 and 0.31 ± 0.19 µmol per µmol of OPGs, respectively (assuming seven glucose residues per molecule of OPG). In agreement with predicted function of succinylation

(Lacorix et al. 1999), *opgC* mutant synthesized OPGs that were devoid of succinate residues. Although *opgB*, *C* and *D* mutants synthesized charged OPGs, there charge distribution was different (Fig. 2a–d). With the application of a step gradient (0.05–0.5 M), OPGs from strain 2457T could be fractionated in two major peaks eluting at 0.05 and 0.1 M NaCl (65% and 32% of total OPGs, respectively) (Fig. 2a). On the other hand, mutation in *opgB* resulted in OPGs that were eluted in a single major peak at salt concentration of 0.05 M (77% of total OPGs) (Fig. 2b). Mutation in *opgC* gene resulted in OPGs with one major peak eluting after application of 0.05 M NaCl, followed by a minor peak when salt concentration was increased to 0.1 M (Fig. 2c). Mutation in *opgD* generated OPGs with heterogeneous charge distribution and required higher salt concentration to discharge from DEAE column (Fig. 2d). In *E. coli*, *opgD* regulates the degree of glucose polymerization, and OPGs from *E. coli* *opgD* mutants had higher degree of polymerization (Lequette et al. 2004). However, we were unable to observe any effect of *opgD* mutation in *S. flexneri*, and influence, if any, on the degree of polymerization as analyzed by MALDI/TOF-MS was minimal (supplemental information, Fig S1). It is not clear at this

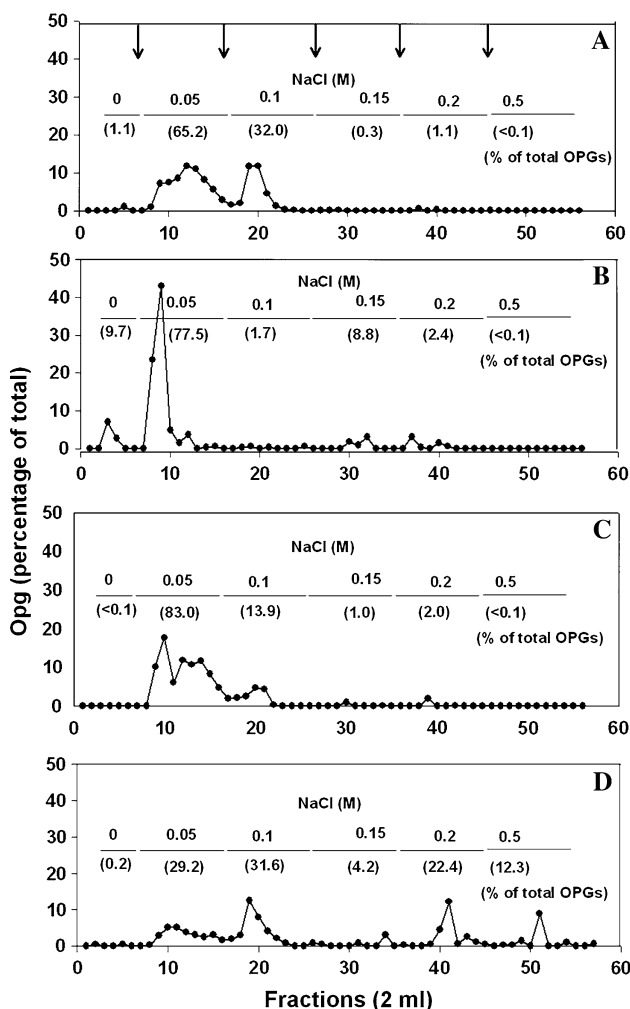


Fig. 2 Fractionation of total OPGs obtained after BioGel P4 chromatography on DEAE-cellulose mini column (1.0 by 1.5 cm). Two mg of total OPGs was dissolved in 250 μ l 7% 1-propanol and applied to column. After eluting neutral OPGs in 10 ml of 7% 1-propanol, step gradient of NaCl in 7% 1-propanol was applied in 20-ml volume (0.05, 0.1, 0.15, 0.2 and 0.5 M NaCl, indicated by arrows) and 2-ml fractions were collected and analyzed for total hexose. Horizontal lines indicate pooled fractions, % OPGs eluted at the indicated NaCl concentration are shown below and above each line, respectively. **a** 2457T; **b** *opgB*; **c** *opgC*; **d** *opgD*

time whether the observed differences reflect variation in OPGs polymerization processes in two organisms or *Shigella* possess yet unidentified redundant information in the genome to substitute for OpgD function, although no paralogs of *opgD* have been reported. *opgD* mutation certainly generated a heterogeneous charge distribution pattern different from other *opg* mutants. Succinate content of OPGs for this mutant was much reduced compared to wild type and may indicate uneven distribution of succinate (or other substituent such as phosphoglycerol) on OPGs. Due to labor intensive protocols and high costs associated with succinic acid determination, individual fractions were not examined for succinic acid contents.

Growth characteristics

When grown in LB medium, time to reach mid-log phase (time to reach 0.5 OD₆₀₀) was 414 ± 3 and 435 ± 6 min for wild-type and *opgGH* strains, respectively (Fig. 3a, open symbols). Osmolarity of LB broth was estimated to be 407 ± 4 mosmol l⁻¹, and at this osmolarity, growth of *opgGH* mutant was closest (but significantly different) compared to wild-type parental strain ($P < 0.05$). Omission of NaCl from LB broth (LBNS) lowered its osmolarity to 85 ± 4 mosmol l⁻¹. In LBNS medium, wild-type strain 2457T entered mid-log phase in 431 ± 7 min after inoculation (a delay of ~ 17 min compared to LB broth), while *opgGH* mutant needed 478 ± 5 min (a delay of ~ 43 min compared to LB broth) ($P < 0.05$) (Fig. 3a, closed symbols). With further reduction in medium osmolarity such as low nutrient low salt medium (LNNS, 36 ± 3 mosmol l⁻¹; Fig. 3b, closed symbols), *opgGH* strain exhibited a much longer lag time in comparison with 2457T (time to reach 0.14 OD₆₀₀; 550 ± 20 vs. 778 ± 31 min, respectively, for 2457T and *opgGH*, $P < 0.001$). The lag period of *opgGH* strain was gradually reduced with increasing medium osmolarity by addition of either NaCl (Fig. 3c, open symbols) or KCl or HEPES buffer (data not shown). Mobilization of pLL3 carrying functional *opgGH* to *opgGH* mutant restored the growth ability of the strain to levels comparable to strain 2457T (Fig. 3b). Mutations in *opgC* and *opgD* but not in *opgB* resulted in longer lag time to initiate growth in hypo-osmotic LNNS growth medium (Fig. 3d). Like *opgGH* mutant, *opgD* mutant responded to changes in osmolarity quickly and restored optimal lag time at mosmol l⁻¹ values 50 and above. On the other hand, *opgC* mutant required medium osmolarity to be 100 mosmol l⁻¹ or more in order to restore optimal lag period. There was >99% gene similarity between *S. flexneri* 2a strain 2457T and *E. coli* *opg* gene family (data not shown). In *E. coli*, *opgB* and *opgC* genes encode enzymes responsible for adding phosphoglycerol and succinate moieties to OPG molecules (Lacorix et al. 1999; Lequette et al. 2008). It is noteworthy that the *opgC* mutant that synthesized OPGs lacking succinate residues also had a suboptimal growth phenotype in hypo-osmotic media (Fig. 3d), while *opgB* mutant that is postulated to be deficient in phosphoglycerate moieties (based on DNA homology to *E. coli* *opgB* gene) generated no detectable growth phenotype.

In vitro characterization of host cell interactions

Several gram-negative plant and animal pathogenic bacteria appear to require OPGs in order to successfully invade respective hosts (Bohin 2000; Bohin and Lacroix 2007). Although precise mechanism by which OPGs assist

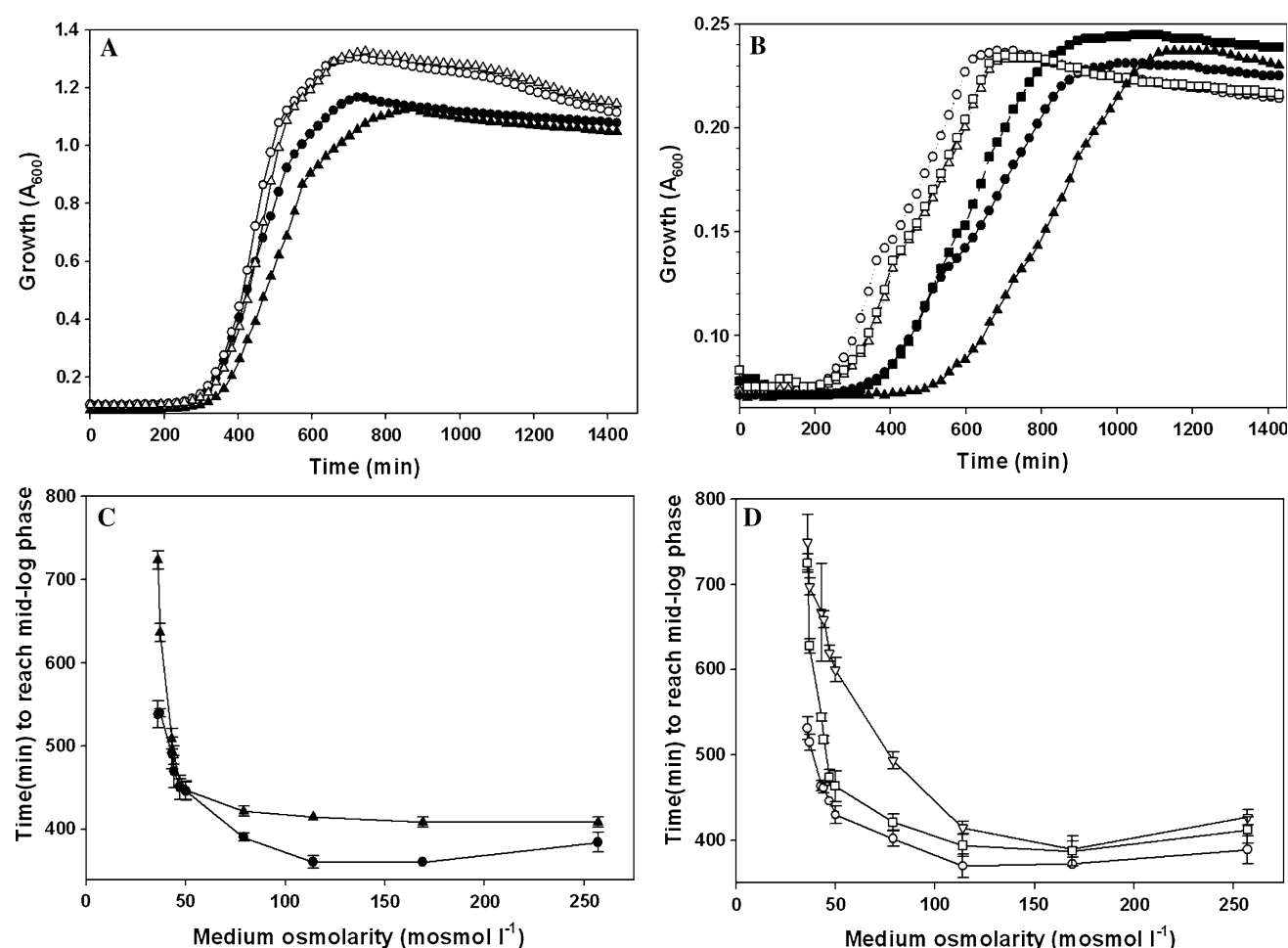


Fig. 3 Growth characteristics of wild-type and *opg* mutants in various osmolarity media. Growth curve kinetics in **a** LBNS (84 mosmol⁻¹; filled circle, filled triangle) and LB (407 mosmol⁻¹; open circle, open triangle) media, 2457T (filled circle, open circle) and *opgGH* (filled triangle, open triangle); **b** LNNS (36 mosmol⁻¹; filled circle, filled triangle, filled square) and LNNS ± 0.155 M NaCl (240 mosmol⁻¹; open circle, open triangle, open square), 2457 T (filled circle, open circle), *opgGH* (filled triangle, open triangle),

opgGH(pLL3) (filled square, open square); **c** and **d** Effect of medium osmolarity on time required to reach mid-log growth phase. LNNS medium was supplemented with varying amounts of NaCl to adjust the osmolarity (36–250 mosmol⁻¹), **c** (filled circle, 2457 T and filled triangle, *opgGH*), **d** (open circle, *opgB*, open square, *opgD*, and open down pointing triangle, *opgC*). Error bars indicate standard deviation of mean values (not shown when smaller than symbol)

pathogens is not known, *Salmonella enterica* serovar Typhimurium mutants defective in OPGs biosynthesis have been shown to be compromised in mice virulence (Bhagwat et al. 2009). Invasion of nonphagocytic host cells is a critical aspect of *Shigella* pathogenesis. The invasion phenotype is mediated by a single type III secretion system (TTSS) encoded on a large invasion plasmid present in all virulent strains of *Shigella*. The TTSS spans both the inner and the outer membrane of the bacterial cell wall; thus, the synthesis of periplasmic OPGs could potentially alter the function of the TTSS. *S. flexneri* 2a *opg* mutants were evaluated for cellular invasion and early cytoplasmic replication using a gentamicin protection assay in HeLa cells. In this assay, intracellular *Shigella* are protected from the effects of gentamicin, which eliminates extracellular bacteria. Each *opg* mutant was recovered from HeLa cells at

levels higher than the parent strain 2457T (Fig. 4). Although *opg* mutants were consistently recovered at levels above the wild-type strain, the difference did not reach statistical significance ($P > 0.05$). Interestingly, *opgGH* mutants of *Salmonella* SG111 and SL_GH3 also were able to invade Caco-2 cells effectively (Fig. 4) in spite of the fact that they lacked the ability to synthesize OPGs and had compromised mice virulence (Bhagwat et al. 2009; Kannan et al. 2009).

Shigella is known to rapidly lyse the endocytic vacuole and spread intracellularly using the *virG* (*icsA*) autotransporter protein to recruit and polymerize host cell actin (Lampel and Maurelli 2001; Day and Maurelli 2002). The presence of comet like actin tails on one pole of the bacteria is a reliable indicator of an intracytoplasmic location and is required for intracellular spread (Fig. 5a). Each *opg*

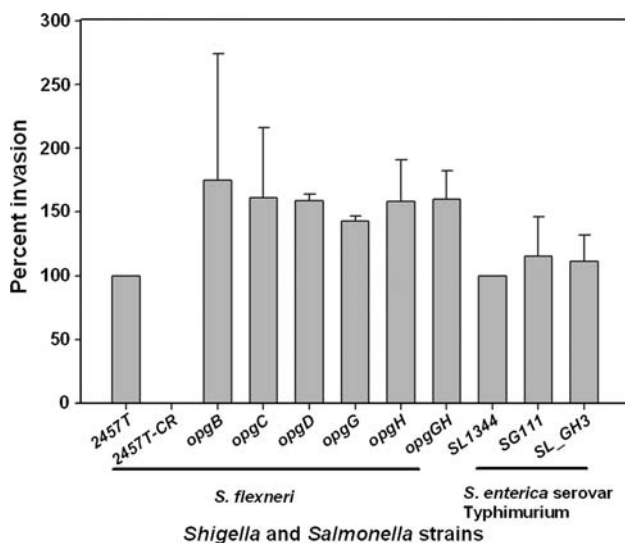


Fig. 4 Invasion of mammalian cell monolayers by *Shigella* and *Salmonella* strains and their *opg* mutants. *Shigella* and *Salmonella* strains were tested for the ability to invade HeLa and Caco-2 cells, respectively, in a gentamicin protection assay. Invasiveness by each *opg* mutant is plotted as a percentage of the invasiveness of respective wild-type strain (2457T for *Shigella* and SL1344 for *Salmonella*) that was set to 100%. 2457T^{CR} indicates a Congo red negative colony. Error bars denote standard deviation of mean values

mutant produced actin tails inside HeLa cells indicating normal actin polymerization after escape into the host cell cytoplasm (Fig. 5b, data not shown for other *opg* mutants).

A plaque assay was then used to monitor not only invasion but also inter- and intracellular spread of wild-type and the *opgGH* mutant. Areas of cell death or plaques can be visualized, by counter staining the BHK cell monolayer for viable cells approximately 72 h after infection. *opgGH* mutants formed plaques of with 0.87 ± 0.35 mm diameter compared to 0.99 ± 0.22 mm diameter observed for wild-type strain. The plaque-forming efficiency was calculated as number of plaques/number of bacteria $\times 100$ at different multiplicity of infections (such as 2.0, 0.2 and 0.02). The plaque-forming efficiency of *opgGH* mutant was 0.009 compared to 0.011 for wild type. Thus, under the in vitro assay conditions, lack of OPGs did not appear to affect bacterial invasion, intracellular replication and dissemination within epithelial cell monolayers (Figs. 4, 5). It may be noted that osmolality of the cell culture media used in vitro cell invasion assays such as MEM with 10% fetal bovine serum, DMEM or RPMI was determined to be in the range of 288–345 mosmol⁻¹ (data not shown). The growth defects of *opgGH*, *opgC* and *opgD* mutants were more pronounced at mosmol⁻¹ value 100 or below (Fig. 3c). In high osmolality media such as LB, growth characteristics of *opg* mutants matched more closely with the wild-type strain. In agreement with this observation, *S. enterica* serovar Typhimurium *opgGH*

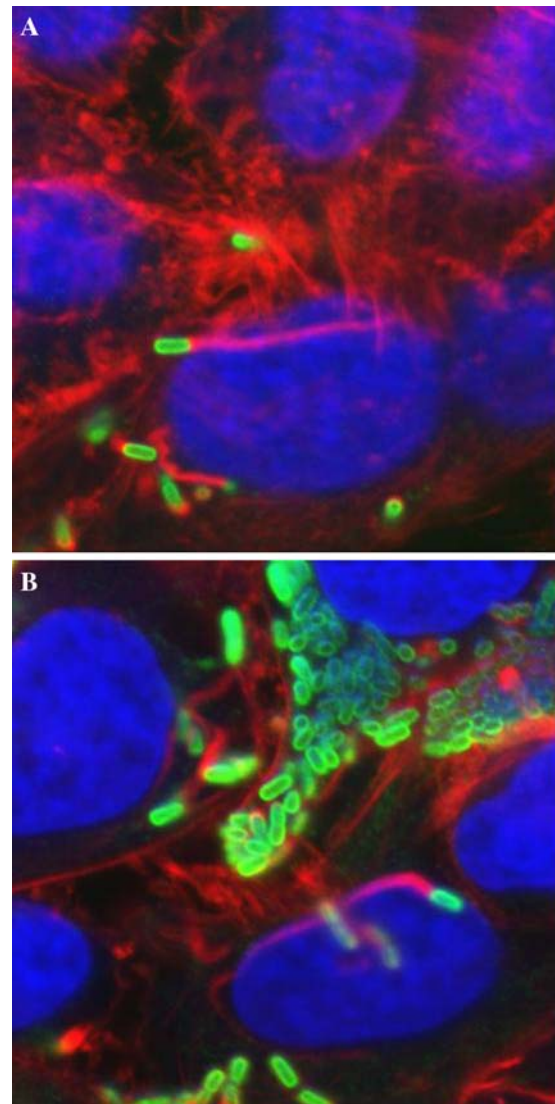


Fig. 5 *virG* (*icsA*)-based actin polymerization inside infected HeLa cells. Both wild-type *S. flexneri* 2457T (a) and *opgGH* (b) were used to infect HeLa cells. Approximately ~4 h after infection and gentamicin treatment, the cells were fixed, permeabilized and stained to visualize actin, bacteria and the nucleus. Evidence of intercellular movement can be visualized by the presence of long comet tails on one pole of the bacterium (magnification $\times 3000$)

mutants although compromised in mice virulence (Bhagwat et al. 2009) were able to infect Caco-2 cell monolayer with no discernable differences with the wild-type strain (Fig. 4). Two distinct phenotypes of *opgGH* mutants of *Salmonella*, suboptimal motility and growth were strictly observed in media with osmolality values <100 mosmol l⁻¹, and normal motility and growth were observed in media with osmolality values >300 mosmol l⁻¹ (Bhagwat et al. 2009; Kannan et al. 2009). Thus, high osmolality of tissue culture media may partially explain why *opg* mutants of *Shigella* and *Salmonella* were able to infect mammalian cells with efficiency comparable to wild-type parents.

This study describes the first report of purification and analysis of OPGs from *S. flexneri* that is a pathogen of significant public health concern. Our data indicate that *S. flexneri* OPGs are needed for optimal growth in low-nutrient, low-osmolarity growth conditions that very likely exist in environments such as irrigation waters and produce wash waters. Succinylation of OPGs appears to be of significant importance since *opgC* mutant although synthesized OPGs, it was defective in hypo-osmotic growth. In vitro invasion studies using various cell lines, however, could not discriminate whether OPGs of *S. flexneri* are required for virulence. Definitive resolution on the role of OPGs in *Shigella* virulence and pathogenesis would require experiments with animal (primate) models.

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